J. Clin. Chem. Clin. Biochem.
Vol. 22, 1984, pp. 175–184

Development of a Chromatographic Method for the Quantitative Determination of Minor Ribonucleosides in Physiological Fluids

Characterization and quantitative determination of minor ribonucleosides in physiological fluids, Part I

By *E. Hagemeier, K. Kemper, K.-S. Boos* and *E. Schlimme*

Laboratorium für Biologische Chemie der Universität (GH) Paderborn, Paderborn, Federal Republic of Germany

(Received October 4/December 1, 1983)

The authors wish to congratulate Professor Walther Lamprecht, Hannover, on the occasion of his sixtieth birthday

Summary: We describe an on-line multi-column high performance liquid chromatographic method for the selective clean-up and analysis of major and minor ribonucleosides in physiological fluids. Quantitative data obtained for the determination of some methylated ribonucleosides in human urines are compared with those obtained with the traditional off-line method.

The on-line technique developed in our laboratory is distinguished from the off-line method by the following features:
(1) Sample clean-up and analysis of the target-compounds can easily be automatized,
(2) Total time of analysis, for example of urinary ribonucleosides, is decreased to 35 minutes,
(3) Laborious and error-prone evaporation and redissolution steps are avoided,
(4) Reliability of the overall analytical system can be controlled with ease,
(5) Small sample-volumes can be applied directly,
(6) Sensitive samples can be processed very rapidly under mild conditions,
(7) Results obtained with the on-line and off-line-techniques compare well.

Entwicklung einer chromatographischen Methode zur quantitativen Bestimmung seltener Ribonucleoside in physiologischen Flüssigkeiten

Charakterisierung und quantitative Bestimmung von seltenen Ribonucleosiden in physiologischen Flüssigkeiten, Teil I

Zusammenfassung: Wir beschreiben ein "on-line"-Mehrsäulen-hochleistungsflüssig-chromatographisches Verfahren zur selektiven Aufreinigung und Analyse häufiger und seltener Ribonucleoside in physiologischen Flüssigkeiten. Die für die quantitative Bestimmung einiger methylierter Ribonucleoside in Humanharnen erhaltenen Ergebnisse werden mit denen eines traditionellen "off-line"-Verfahrens verglichen.

Im Vergleich zum off-line Verfahren zeichnet sich die von uns entwickelte "on-line"-Technik durch folgende Eigenschaften aus:
(1) Probenaufreinigung und Analyse der Zielsubstanzen lassen sich leicht automatisieren,
(2) Die Gesamtanalysendauer, z. B. für harngängige Ribonucleoside verkürzt sich auf 35 Minuten,
(3) Aufwendige und fehlerträchtige Konzentrierungs- und Wiederaufnahmeschritte entfallen,
(4) Die Zuverlässigkeit des gesamten analytischen Systems kann mühelos kontrolliert werden,

(5) Kleine Probenvolumina können direkt appliziert werden,
(6) Empfindliche Proben können in kürzester Zeit unter schonenden Bedingungen analysiert werden,
(7) Die mit der "on-line" wie mit der "off-line"-Technik erhaltenen Ergebnisse zeigen eine gute Übereinstimmung.

Introduction

The measurement of major and minor (modified) nucleosides (fig. 1) in biological fluids, such as urine or serum or in hydrolysates of RNA and DNA, has become of potential interest in molecular biology and clinical biochemistry. As constituents of mRNA, rRNA and especially of tRNA, modified nucleosides play a significant role in protein synthesis as well as in many other regulatory processes (1, 2). After breakdown of RNA the modified nucleosides, in contrast to major nucleosides, do not enter the salvage pathways (3) but are excreted almost entirely in urine (4, 5); they are thus powerful indicators of pathobiochemical processes, e.g. tumour growth (6), adenosine deaminase deficiency and severe combined immunodeficiency (7, 8) or lack of hypoxanthine/guanine phosphoribosyltransferase activity (*Lesh-Nyhan* syndrome) (3).

Taking into account that more than 50 modified nucleosides have been isolated and characterized in tRNA (9) and more than 30 in urine (10) the necessity for the development of a sensitive and efficient analytical method becomes obvious.

Whereas in recent years much effort has been put into the improvement of resolution, sensitivity of detection and reliability of the chromatographic system in nucleoside analysis (11–13), the techniques for sample preparation, however, have not kept pace with these developments.

Boronate gel affinity chromatography for the clean up of ribonucleosides in biological fluids, introduced in 1976 by *Uziel, Smith & Taylor* (14) and optimized by *Davis* and co-workers (15), still presents the generally preferred technique for the clean-up of ribonucleosides in urine or serum (16–21).

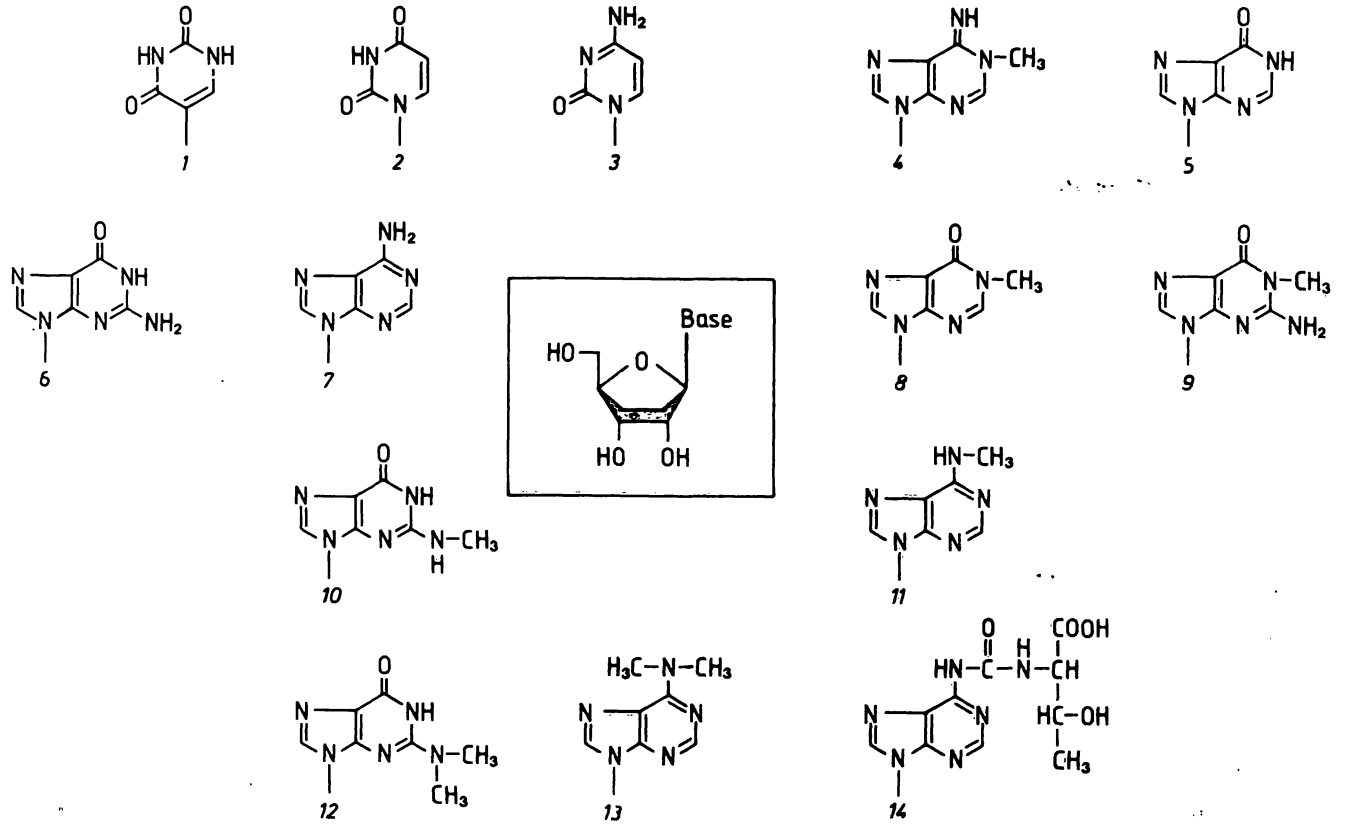


Fig. 1. Ribonucleosides investigated.
1 Pseudouridine (Ψ)
2 Uridine (Urd)
3 Cytidine (Cyd)
4 N1-methyladenosine (m^1Ado)
5 Inosine (Ino)
6 Guanosine (Guo)
7 Adenosine (Ado)
8 N1-methylinosine (m^1Ino)
9 N1-methylguanosine (m^1Guo)
10 N2-methylguanosine (m^2Guo)
11 N6-methyladenosine (m^6Ado)
12 N2-dimethylguanosine (m^2_2Guo)
13 N6-dimethyladenosine (m^6_2Ado)
14 N6-(carbamoyl-threonyl)adenosine (t^6Ado).

Encouraged by the publication of a boronic acid-functionalized silica (22), which is particular suitable for HPLC, we attempted to transfer the commonly used urinary clean-up procedure from low pressure column chromatography to HPLC conditions thus applying a method known as high-performance liquid (ligand) affinity chromatography (HPLAC) (23). As the material described was not suitable for this purpose we developed a new boronate silica which fulfilled the conditions required (24). By coupling the HPLAC system, developed in our laboratory, to reversed-phase HPLC (RP-HPLC) by a column switching technique, we finally evolved a chromatographic system for the on-line clean-up and analysis of ribonucleosides in physiological fluids (25, 26).

In the present paper this on-line method is compared with the traditional off-line procedure.

Experimental

Materials and Methods

Chemicals

N1-methyladenosine (m^1Ado), N1-methylinosine (m^1Ino), N1-methylguanosine (m^1Guo), N2-methylguanosine (m^2Guo), N6-dimethyladenosine (m^6_2Ado), N6-methyladenosine (m^6Ado), pseudouridine (Ψ) were purchased from Sigma, München. Adenosine (Ado), inosine (Ino), guanosine (Guo), uridine (Urd) and cytidine (Cyd) were from Boehringer, Mannheim. N2-dimethylguanosine (m^2_2Guo) and N6-(carbamoyl-threonyl)-adenosine (t^6Ado) were isolated from urine and characterized by UV-, mass- and ^{1}H-NMR-spectroscopy as well as by characteristic chemical reactions; for further characterization t^6Ado was chemically synthesized in parallel (27).

In all buffer preparations double distilled water and salts of purest grade available were used. Organic solvents were of LiChrosolv quality (Merck, Darmstadt).

Synthesis of affinity supports

Boronic acid-functionalized polyacrylamide was synthesized by coupling Hydrazide-Bio-Gel P-2 (200–400 mesh, hydrazide capacity 2.4 mmol/l per dry gram, Bio-Rad, München) with succinic anhydride and *m*-aminobenzeneboronic acid (Sigma) according to l. c. (14, 15).

For synthesis of boronic acid-functionalized silica LiChrosorb Si 100, 5 µm, (Merck) was substituted with γ-chloropropyltrimethoxysilane (generous gift from Dynamit Nobel, Troisdorf) and subsequently reacted with *m*-aminobenzeneboronic acid according to l. c. (24). Meanwhile a boronic acid substituted silica of comparable quality is commercially available from Serva Feinbiochemica, Heidelberg as "Dihydroxyboryl-Polyol Si 100", 5 µm.

HPLC apparatus

The basic HPLC equipment consisted of two Altex Model 110 A pumps (Altex, USA) controlled by a Model 420 microprocessor, a Rheodyne Model 7125 loop injector for sample introduction and a Kontron Uvicon Model 725 spectrophotometer (Kontron, Eching) for monitoring the UV-absorbance of the effluent at 259 nm. Areas under the peaks obtained were integrated with a Hewlett Packard Model 3390 A integrator (Hewlett-Packard, Frankfurt).

For on-line analysis this basic gradient system was additionally equipped with a third Altex Model 110 A pump, a second Rheodyne 7125 loop injector and a Rheodyne Model 7010 six-port valve, which were incorporated as shown schematically in scheme 1 (top).

Sample collection and storage

24 h urine specimens were stored at 0–5 °C with sodium azide as preservative until collection was completed. After collection, 20 ml portions were adjusted to pH 4 with 850 g/kg formic acid and stored at −20 °C.

Fresh blood samples were centrifuged at 2000 *g* for 10 minutes at 4 °C. Serum was collected, adjusted to pH 4 with 850/kg formic acid and subsequently stored at −20 °C or immediately processed.

Analytical procedure

Off-line method

1) Sample preparation

For analysis, 1.5 ml of urine was mixed with 150 µl of 2.5 mol/l ammonium acetate pH 8.8 and, if necessary, with 100–200 µl 2 mol/l sodium hydroxide to obtain a slightly alkaline pH (pH 8). Urine samples were then membrane filtered (Millox 0.22 µm, Millipore, France) and immediately applied to the affinity gel column.

Human serum (1.5 ml) was deproteinized within 40 min by centrifugal ultrafiltration (Amicon, Micropartition System) at 5000 *g* and 4 °C. After pH adjustment, 1 ml of serum ultrafiltrate was applied to the affinity gel column.

2) Clean-up of ribonucleosides on boronic acid-substituted affinity gel

The selective group separation of ribonucleosides in urine or serum ultrafiltrate was achieved by ligand affinity chromatography using a glass column (50 × 5 mm I.D.) filled with boronic acid-functionalized Bio-Gel P-2. Elution was carried out with a Model Minipuls II pump (Gilson, France). The effluent was monitored continuously with an ISCO Model UA 5 absorbance detector (Instrumentation Specialties, USA) at 254 nm. The affinity gel column was equilibrated at pH 8.8 with 0.25 mol/l ammonium acetate. The sample (1 ml of pretreated urine, serum ultrafiltrate or synthetic nucleoside mixture) was transferred to the top of the boronate gel column, which was then eluted successively with

(A) 0.25 mol/l ammonium acetate pH 8.8 (fraction 1),

(B) H_2O (fraction 2) and

(C) 0.1 mol/l formic acid (fraction 3).

Fractions 2 and 3 were shell frozen, lyophilized and redissolved in 2 × 500 µl of H_2O. Aliquots were then analysed by HPLC.

3) HPLC determination of nucleosides

The column used for RP-HPLC of fraction 2 was a stainless steel column, 125 × 4 mm I.D., packed in the laboratory with Nucleosil® 5C18 (Macherey & Nagel, Düren). Cation-exchange chromatography of fraction 3 was run on an Altex Partisil SCX column, 10 µm (250 × 3.2 mm I.D.).

Chromatographic conditions are specified in legends to the figures.

On-line method

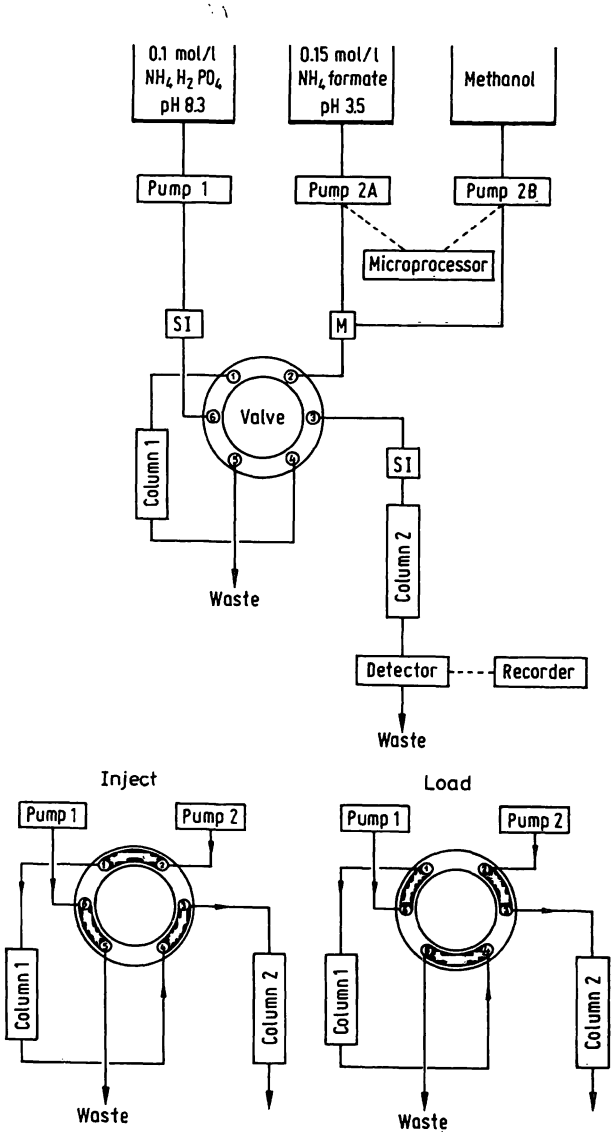
1) Sample preparation

Human urine (500 µl) was membrane filtered (Millox 0.22 µm) and an aliquot of 50 µl was applied to the HPLAC-column. Samples of human serum (250 µl) were deproteinized within 10 min by centrifugal ultrafiltration at 5000 *g* and 4 °C. The appropriate ultrafiltrate (50 µl) was applied to the HPLAC-column.

2) On-line clean-up and analysis of ribonucleosides in urine and serum

For the on-line analysis of ribonucleosides in urine or serum the HPLAC-column 1 (30 × 4 mm I.D., filled with boronic acid-substituted silica) was equilibrated for 2 min in valve position "Load" with 0.1 mol/l ammonium phosphate pH 8.3. After sample injection column 1 was washed with the same buffer for 2 min. During that time ribonucleosides were selectively retarded on the HPLAC-column whereas the sample matrix was discharged.

After this clean-up step the valve was switched to "Inject" and thereby series-connected in front of column 2 (250 × 4 mm I.D., filled with LiChrosorb RP 18, 7 µm, Merck). The group specifically bound ribonucleosides on column 1 were then eluted under acidic conditions (0.15 mol/l ammonium formate pH 3.5) in a small volume through positions 2–1–4–3 of the valve (Compare scheme I) and concentrated on top of column 2 over a period of 1.5 min. This elution buffer was also used for the subsequent analytical separation. The valve was then switched back into position "Load" and elution of column 2 could be carried out independently by increasing the amount of organic modifier in the mobile phase. Chromatographic conditions are given in legends to the appropriate figures.



Scheme 1 Apparatus.
Top: On-line system setup
Bottom: Valve switching positions.

Quantitative determinations

Calibration mixtures for quantitative evaluation of the nucleoside peaks obtained by HPLC were prepared by dissolving 2 mg of the appropriate nucleoside in 1 ml of double distilled water or, in the case of m^1Ado, in 0.1 mol/l formic acid. As some nucleosides do not dissolve entirely, the solutions were centrifuged at 2000 *g* for 2 min and the supernatants transferred to testtubes. The nucleoside concentration in the supernatant was then determined spectrophotometrically (Zeiss PMQ 3). The final concentration was calculated as the mean of three independent analyses. For calibration of the chromatographic system according to the "External Standard Method" calibration mixtures containing 200 to 400 pmol each of the appropriate nucleosides were analysed and absolute molar factors for each nucleoside were determined by the integrator as the mean value of at least four independent runs. Calibration was checked daily and recalibration was carried out if the calculated amounts for the calibration mixture differed more than 5% from the standard values.

Results and Discussion

Off-line clean-up and analysis of ribonucleosides in physiological fluids

Affinity chromatography of ribonucleosides on boronic acid-functionalized polyacrylamide gel

Boronic acid is known to form complexes generally with 1,2 cis-diol bearing compounds as present in the ribose moiety of nucleosides, various carbohydrates or catechols (28, 29) (fig. 2).

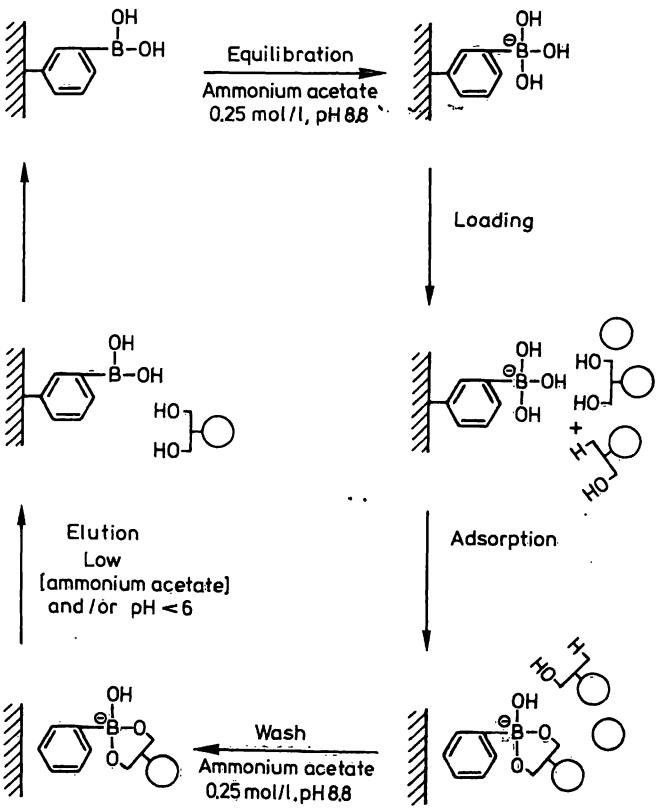


Fig. 2: Boronic acid affinity chromatography based on pH-shift.
O, O–[H/OH, O–[OH/OH = substances without and with cis-diol groups

The target molecules selectively bound on the boronate gel are eluted by lowering the buffer salt concentration or decreasing the pH of the mobile phase – both being conditions which lead to a destabilization of the boronate-cis-diol complex (29). By applying this two step gradient elution we could achieve a prefractionation of urinary and serum ribonucleosides in which "neutral" nucleosides such as Ino, Guo, Ado, m^1Ino, m^2Guo etc. are eluted with H_2O; whereas the cationic ones, for example m^1Ado and m^7Guo, elute with formic acid (cf. fig. 3).

Fractions 2 and 3 were lyophilized, redissolved in defined volumes and aliquots further investigated by HPLC.

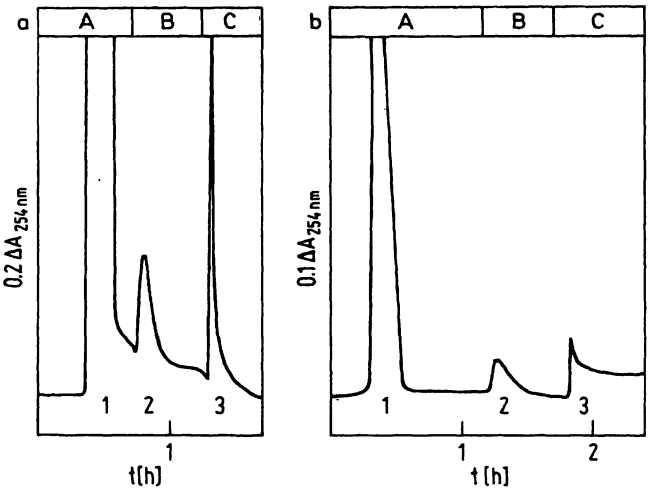


Fig. 3. Typical elution profiles from a boronic acid affinity gel column.
The samples of
a) 1.0 ml pretreated (cf. Analytical procedure, Off-line method, Sample preparation) urine or
b) 1.0 ml serum ultrafiltrate were eluted with
(A) 0.25 mol/l ammonium acetate pH 8.8 (fraction 1),
(B) H_2O (fraction 2) and
(C) 0.1 mol/l formic acid (fraction 3).
Fraction 1 consists of non retarded substances, fraction 2 and 3 contain the neutral and cationic ribonucleosides, respectively.

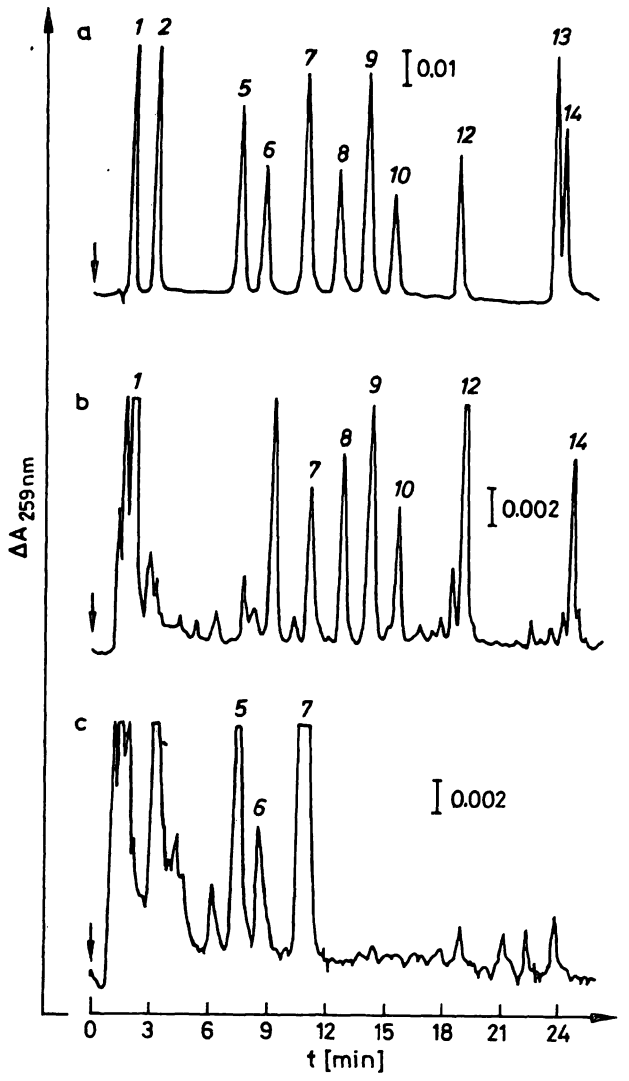


Fig. 4. Reversed-phase HPLC separation of ribonucleosides in physiological fluids.
a) Separation of a synthetic mixture (100 µl; 2–10 nmol of each nucleoside). The peak numbering refers to figure 1.
b) Separation of a 100 µl aliquot of a "water fraction" obtained in the liquid chromatographic prefractionation step of urine (cf. Results and Discussion, Off-line clean-up, ..., Affinity chromatography ...). The nucleosides *8*, *10*, *12* were quantitatively investigated.
c) Separation of a 100 µl aliquot of a "water fraction" obtained by the prefractionation procedure of a serum sample. Prior to prefractionation the sample was concentrated 20-fold.
Chromatographic conditions: Nucleosil® 5 C 18 (125 × 4 mm I.D.), buffer: 0.2 mol/l ammonium formate pH 3.5; elution: 0–5 min isocratically with methanol-free buffer, 5–11 min with buffer/methanol (96 + 4 by volume), after 11 min a gradient was run up to buffer/methanol (70 + 30 by volume) in 18 min; flow rate: 1.3 ml/min; detection: 259 nm.

HPLC-analysis of ribonucleoside fractions

Fraction 2 obtained in the clean-up procedure was analysed by RP-HPLC. A detailed description of parameters affecting the separation of major and modified nucleosides in the reversed-phase mode of HPLC is given in l. c. (12).

Figure 4 shows in a) the separation of a synthetic mixture of 11 naturally occurring ribonucleosides; in b) the separation of a 100 µl aliquot of a "water" fraction (fraction 2) obtained in a prefractionation step of urine.

Although peak *7* (retention time identical with Ado) and peak *9* (retention time identical with m^1Guo) appear as single peaks, it could be demonstrated by variation of the pH or ionic strength of the mobile phase that these compounds coelute with at least one

or two other UV-absorbing cis-diol bearing compounds. As no satisfactory resolution could be achieved continuously, peaks *7* and *9* were not considered for quantitative investigations. Peaks *8*, *10* and *12* were characterized as m^1Ino (*8*), m^2Guo (*10*) and m_2^2Guo (*12*) by comparison of retention times of authentic synthetic nucleosides and co-chromatography of authentic nucleosides in a spiked control urine. To prove homogeneity of the chromatographic zone, peaks *8*, *10* and *12* were frequently pooled during the analyses of "water"fractions and additionally characterized by UV-, mass- and ^{1}H-spectroscopy as well as specific chemical reactions (27).

Figure 4c shows the RP-HPLC separation of a "water"fraction obtained in the clean-up of human serum ultrafiltrate. Ino (*5*), Guo (*6*) and Ado (*7*) could be characterized as main components by enzymatic peak shift and quench, respectively.

The quantitative determination of these major nucleosides in serum samples, however, is difficult because enzyme activities are present in native serum. Thus, for example, adenosine is quantitatively deaminated to inosine within 3 h after venipuncture. Inosine, on the other hand, is converted to hypoxanthine, although at a slower rate (11).

By adjusting serum to pH 4 immediately after centrifugation we were able to suppress enzyme activities so that adenosine, guanosine and inosine could be characterized at least qualitatively (30). The minor ribonucleosides in human serum still present analytical difficulties as they are present only in very small amounts. Preliminary results indicate m^1Ino, m^2Guo and m_2^2Guo concentrations of about 100 nmol of each nucleoside per liter of human serum (31).

The "acidic" fraction (fraction 3; urine) was analysed by ion-exchange HPLC as outlined in l. c. (17). The main component of this fraction was characterized as m^1Ado, accompanied by small amounts of m^7Guo. The identity of m^1Ado was confirmed by characteristic chemical reactions and chromatographic properties (17, 32) and further considered in our quantitative investigations (33).

Accuracy and precision of urinary ribonucleoside determination

The reliability for the off-line procedure has been frequently proved (14–16). Matrix-dependent and -independent analyses of the nucleosides investigated gave recoveries > 90% (Average standard deviations for the determination of m^1Ado, m^1Ino, m^2Guo and m_2^2Guo are given in table 3).

On-line clean-up and analysis of ribonucleosides in physiological fluids

On-line analysis

With the development of a HPLAC-column (24) the aforementioned low pressure liquid chromatography (LAC) technique can now be performed under high pressure conditions. Under acidic conditions, the ribonucleosides investigated elute from the HPLAC-column in a small volume (approx. 700 µl). This allows a column switching technique, i.e., the direct transfer of this zone to a reversed-phase column. In addition, most nucleosides are concentrated on top of the RP-C_{18} column due to their hydrophobic character. After this transfer-step, RP-HPLC can be run independently and elution of ribonucleosides can be carried out by increasing the amount of organic modifier in the mobile phase (fig. 5).

A comparison of the RP-HPLC run (fig. 5a) and the on-line procedure (fig. 5b) shows that the latter does not affect bandbroadening and resolution of the nucleosides investigated. This result is in good agreement with the findings of *Gehrke* et al. (12) who demonstrated that sample volumes up to 1 ml do not essentially influence theoretical plate height and resolution of nucleosides on a RP-C_{18} column.

The method described allows the direct analysis of ribonucleosides in native urine or deproteinized serum within 33 min with a detection limit of 1 µmol of the appropriate nucleoside per liter biological fluid.

Accuracy and precision of urinary ribonucleoside determination

1) Linearity

Figure 6 shows the excellent linearity between sample volume analysed and amount of nucleoside found. Linearity was proved for the determination of m^1Ado, m^1Ino, m^2Guo and m_2^2Guo in 20 to 100 µl of native urine. Between 100 and 2500 pmol of the appropriate nucleoside could be measured with correlation coefficients for linear regression between 0.994 and 1.003. The wide linear range is sufficient for routine determination and analysis of nucleosides in physiological fluids.

2) Analytical recovery of nucleosides

To monitor the accuracy of the overall chromatographic system, the matrix-dependent and -independent recovery of nucleosides was determined. For matrix-independent recovery a synthetic mixture of nucleosides (*4*, *8*, *10*, *12*) was applied directly to the RP-column (compare Scheme I) or analysed

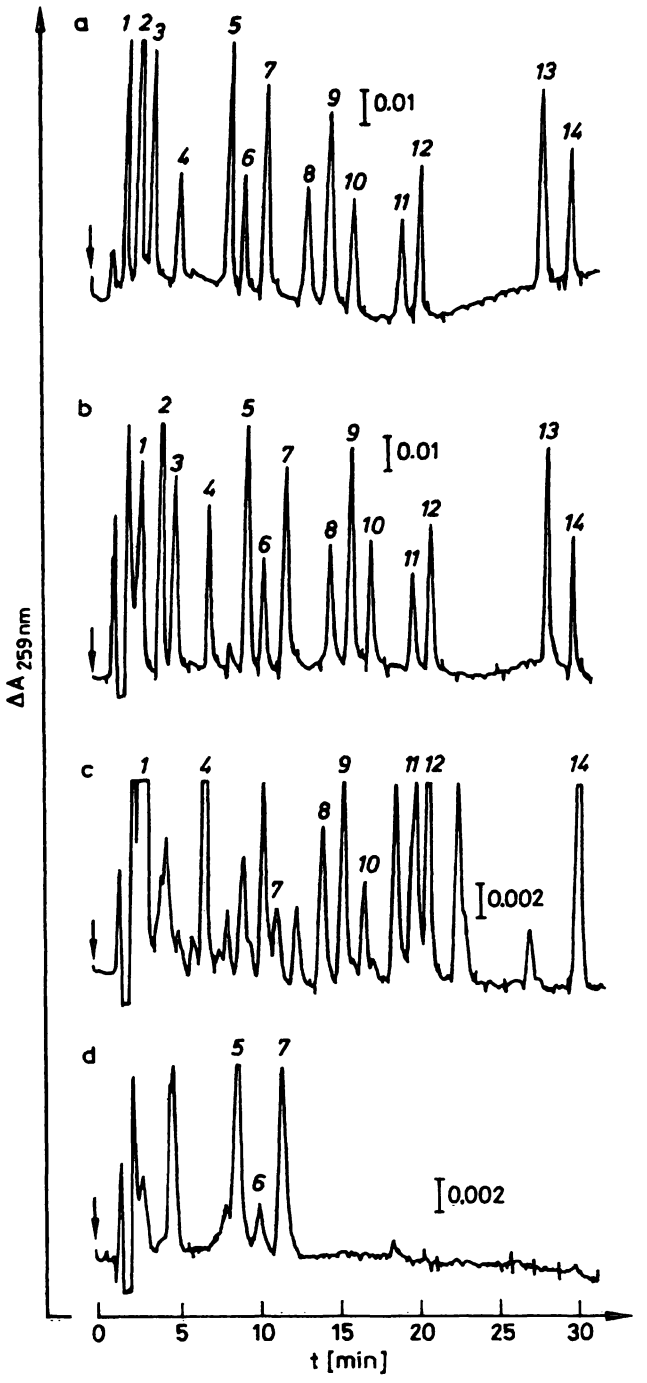


Fig. 5. HPLAC/Reversed-phase HPLC separation of ribonucleosides.
a) Off-line (HPLC) chromatogram of 14 synthetic major and minor ribonucleosides (100 µl; 2–10 nmol of each nucleoside).
b) On-line (HPLAC-HPLC) chromatography of a 100 µl sample of the same nucleoside mixture as above.
c) On-line chromatography of a urine sample (50 µl).
d) On-line chromatography of a serum ultrafiltrate (50 µl).
The elution of column 1 in the on-line system is described in detail in section Analytical procedure, On-line-method, On-line clean up ...
Chromatographic conditions for column 2: Li Chrosorb RP 18, 7 µm (250 × 4 mm I.D.); elution: 0.15 mol/l ammonium formate pH 3.5; after 2 min a linear gradient was run up to ammonium formate/methanol (92 + 8 by volume) in 10 min followed by a linear gradient up to ammonium formate/methanol (70 + 30 by volume) in 20 min.

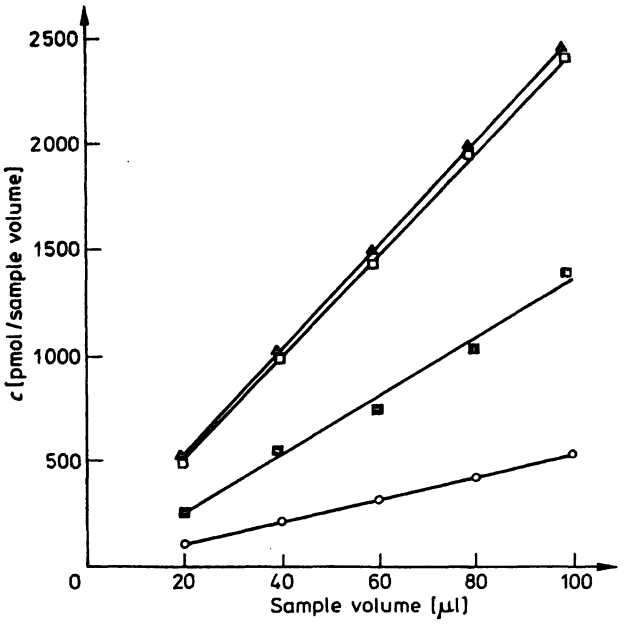


Fig. 6. Linearity of on-line HPLAC/RP-HPLC analysis of m^1Ado, m^1Ino, m^2Guo and m^2_2Guo.
m^2_2Guo (▲–▲), r = 1.003; m^1Ado (□–□), r = 0.999; m^1Ino (■–■), r = 0.994; m^2Guo (○–○), r = 0.999.

by the on-line HPLAC/RP-HPLC system. For matrix-dependent recovery the amount of nucleoside present in a control urine was determined by the External Standard Method. The control urine was then spiked with defined amounts of nucleosides and analysed anew. Results are summarized in tables 1 and 2.

Tab. 1. Matrix-independent recovery of nucleosides.

Nucleoside	Recovery (%)	CV (%)
m^1Ado	99.4[a]	1.3[b]
m^1Ino	99.7	1.6
m^2Guo	97.0	3.6
m^2_2Guo	99.8	4.3

[a] Each value is an average of three runs
[b] Relative standard deviation

Tab. 2. Matrix-dependent recovery of nucleosides added to pooled control urine.

Nucleoside	Concentration[a] (µmol/l) Urine	Spike	Urine + Spike (found)	Average recovery (%)
m^1Ado	11.49	5.17	16.41 ± 0.3[b]	99
m^1Ino	7.46	9.91	17.03 ± 0.2	98
m^2Guo	3.48	5.40	8.88 ± 0.1	100
m^2_2Guo	9.91	4.04	13.65 ± 0.3	98

[a] Each value is an average of three runs
[b] Standard deviation

Off-line/on-line comparison

Figure 7 representatively shows the good correlation of results obtained by both the off-line and on-line method for the determination of m^1Ado in 25 different urines (values for m^1Ino, m^2Guo and m^2_2Guo are given in legend to figure 7).

Whereas the regression line for m^1Ado nearly corresponds with the bisector of the angle indicating high concurrence of the results obtained by both methods, the regression lines for m^1Ino (*8*), m^2Guo (*10*) and m^2_2Guo (*12*) show a slightly lower slope. This may partly be explained by the fact that the on-line method gave better analytical recovery of nucleosides than the off-line method.

Generally both methods turn out to be appropriate for the determination of ribonucleosides in physiological fluids. Figure 8, however, illustrates the considerable advantages of the on-line method.

(1) Total time of analysis is tremendously shortened. Sample clean-up, including time-consuming evaporation steps, is no longer a time limiting factor and is decreased from 145 to 2 min.

(2) Small sample volumes can be analysed. As far as human serum is concerned only 150 to 250 µl of na-

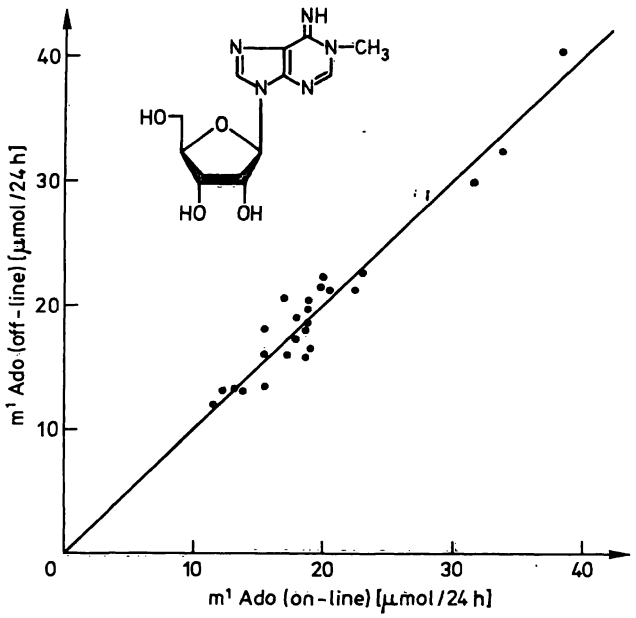


Fig. 7. Off-line/one-line correlation.
The comparison of the "off-line" and "on-line"-chromatography methods is based on the quantitative determination of m^1Ado, m^1Ino, m^2Guo and m^2_2Guo in 20 different urines and representatively shown for m^1Ado.
Each value is an average of three independent runs. Regression lines:
m^1Ado, y = 1.003 x + 0.006, r = 0.9633;
m^1Ino, y = 0.894 x + 2.132, r = 0.9185;
m^2Guo, y = 0.642 x + 1.515, r= 0.6988;
m^2_2Guo, y = 0.937 x + 0.178, r = 0.9385.

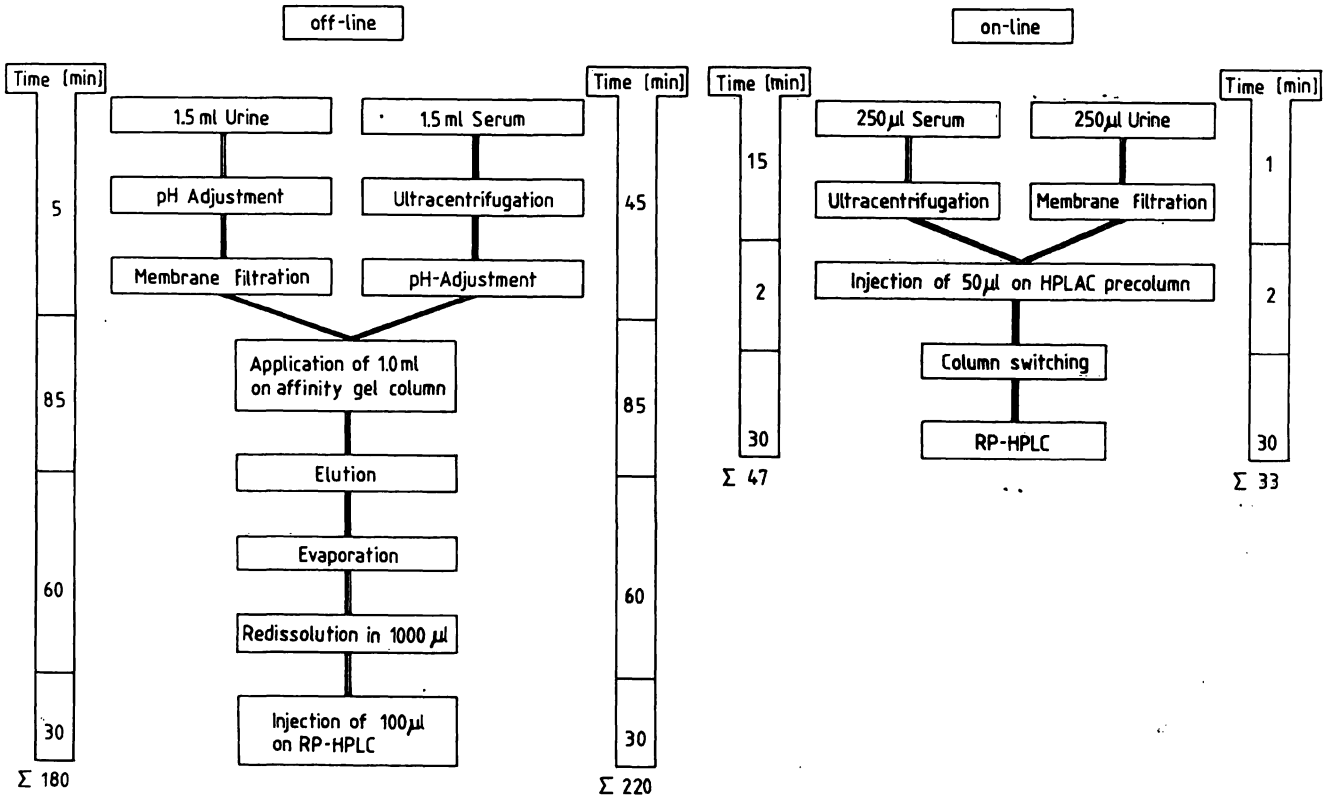


Fig. 8. Flow diagram.
The comparison of the off-line and the on-line method shows in detail the working steps and the time advantage gained by the on-line system.

tive serum must be deproteinized by ultracentrifugation for determination of major nucleosides, i.e. this procedure reduces sample processing costs.

(3) Sensitive samples, e.g. human serum can be processed very rapidly.

(4) Small sample volumes, for example micropunctuates or RNA-hydrolysates, can be directly applied and analysed.

(5) The apparatus allows easy automatization by use of an automatic sample processor and a pneumatic switching valve (compare Scheme I).

(6) The analytical procedure can be easily controlled for its reliability.

(7) Error-prone evaporation- and redissolution steps are avoided, thus leading to an improvement of the analytical precision. Table 3 shows the average coefficient of variation (%) for the determination of m^1Ado, m^1Ino, m^2Guo and m_2^2Guo in 25 different urines.

Tab. 3. Average relative standard deviation (CV, %) for the determination of m^1Ado, m^1Ino, m^2Guo and m_2^2Guo in 25 different urines.

Nucleoside	Off-line	On-line
m^1Ado	8.4[a]	2.7[a]
m^1Ino	7.4	3.6
m^2Guo	8.6	7.9
m_2^2Guo	6.8	2.6

[a] Relative standard deviation. Each of the 25 urine values was determined three times.

Conclusions

The on-line HPLAC/RP-HPLC method described in this paper is compared with the conventional off-line LAC/RP-HPLC procedure which was applied by us for the quantitative determination of m^1Ado, m^1Ino,m^2Guo and m_2^2Guo in urines of pregnant and non-pregnant females (33). The results obtained by both methods show good concurrence and correlation. Since the occurrence of nucleosides in physiological fluids turned out to be predicative for the diagnosis of pathobiochemical processes, the on-line system is the method of choice, as it is particularly suitable for automatization and applicable for routine clinical analysis. As affinity chromatography on boronic acid-functionalized polymers is also well known for the pre-purification of catecholamines in physiological fluids (34–38), for the separation of ribonucleosides and ribonucleotides from the corresponding deoxy-compounds (22, 39, 40), the separation of RNA from DNA (41, 42) and the separation of queuine-containing tRNA from non-queuine tRNA (41) or aminoacyl-tRNA from uncharged tRNA (41, 42) the use of boronate HPLAC in combination with the presented column switching technique offers a wide range of application.

References

1. Kerr, S. J. & Borek, E. (1972) Adv. Enzymol. *36*, 1–27.
2. McCloskey, J. A. & Nishimura, S. (1977) Acc. Chem. Res. *10*, 403–410.
3. Murray, A. W. (1971) Ann. Rev. Biochem. *50*, 811–826.
4. Mandel, L., Srinivasan, P. & Borek, E. (1966) Nature *209*, 586–588.
5. Dlugajczyk, A. & Eiler, J. (1966) Nature *212*, 611–612.
6. Recent Results in Cancer Research: Modified Nucleosides and Cancer (Nass, G., ed.) Springer Verlag, Berlin-Heidelberg-New York (1983).
7. Hartwick, R. A. & Brown, P. R. (1977) J. Chromatogr. *143*, 383–389.
8. Hirschhorn, R., Ratech, H., Rubinstein, A., Papageorgiou, P., Kesarwala, H., Gelfand, E. & Roegner-Maniscalco, V. (1982) Pediatr. Res. *16*, 362–369.
9. Dunn, D. B. & Hall, R. in: Handbook of Biochemistry and Molecular Biology: Nucleic Acids, 3rd ed. (Fasman, G., ed.) CRC Press, Cleveland, Ohio (1975).
10. Chheda, G. B., o. c. (9), 251–270.
11. Hartwick, R. A., Assenza, S. P. & Brown, P. R. (1979) J. Chromatogr. *186*, 647–658.
12. Gehrke, C. W., Kuo, K. C. & Zumwalt, R. W. (1980) J. Chromatogr. *188*, 129–147.
13. Assenza, S. P. & Brown, P. R. (1980) J. Liq. Chromatogr. *3*, 41–59.
14. Uziel, M., Smith, L. H. & Taylor, St. A. (1976) Clin. Chem. *22*, 1451–1455.
15. Davis, G. E., Suits, R. D., Kuo, K. C., Gehrke, C. W., Waalkes, T. P. & Borek, E. (1977) Clin. Chem. *23*, 1427–1435.
16. Gehrke, C. W., Kuo, K. C., Davis, G. E., Suits, R. D., Waalkes, T. P. & Borek, E. (1978) J. Chromatogr. *150*, 455–476.
17. Schlimme, E., Boos, K.-S. & Weise, M. (1981) J. Clin. Chem. Clin. Biochem. *19*, 55–59.
18. Pfadenhauer, E. H. & Tong, S. D. (1979) J. Chromatogr. *162*, 585–590.
19. Schöch, G., Thomale, J., Lorenz, H., Suberg, H. & Karsten, U. (1980) Clin. Chim. Acta *108*, 247–257.
20. Rasmusson, T., Björk, G. R., Damber, L., Holm, S. E., Jacobsson, L., Jepsson, A., Littbrand, B., Stigbrand, T. & Westman, G. (1983) in o. c. (6).
21. Colonna, A., Russo, T., Cimino, F. & Salvatore, F. (1981) J. Clin. Chem. Clin. Biochem. *19*, 640.
22. Glad, M., Ohlson, S., Hansson, L., Mansson, M. O. & Mosbach, K. (1980) J. Chromatogr. *200*, 254–260.
23. Ohlson, S., Hansson, L., Larsson, P. O. & Mosbach, K. (1978) FEBS Lett. *93*, 5–9.
24. Hagemeier, E., Boos, K.-S., Schlimme, E., Lechtenbörger, K. & Kettrup, A. (1983) J. Chromatogr. *268*, 291–295.

25. Hagemeier, E., Boos, K.-S., Lechtenbörger, K. & Schlimme, E. (1982) Fresenius Z. Anal. Chem. *311*, 421.
26. Hagemeier, E., Kemper, K., Boos, K.-S. & Schlimme, E. (1983) J. Chromatogr. *282*, 663–669.
27. Kemper, K., Michels, W., Hagemeier, E., Boos, K.-S. & Schlimme, E. (1983) unpublished results.
28. Böeseken, J. (1949) Adv. Carbohyd. Chem. *4*, 189–210.
29. Fulton, S. (1981) in: Boronate Ligands in Biochemical Separations (Amicon Corporation, ed.), Studio 6, Inc. Sudburry, MASS., USA.
30. a) Barth, S. (1982) Staatsexamensarbeit, Univ. Paderborn. b) Meyer, U. (1984) Staatsexamensarbeit, Univ. Paderborn.
31. Gehrke, C. W., Zumwalt, R. W., Mc Cune, R. A. & Kuo, K. C. (1983) in o. c. (6).
32. Schlimme, E., Hagemeier, E., Boos, K.-S. & Weise, M. (1980) J. Clin. Chem. Clin. Biochem., *18*, 696–697.
33. Hagemeier, E., Kemper, K., Boos, K.-S., Schlimme, E. & Weise, M. (1984) J. Clin. Chem. Clin. Biochem., in preparation.
34. Higa, S., Suzuki, T., Hayashi, A., Tsuge, I. & Yamamura, Y. (1977) Anal. Biochem. *77*, 18–24.
35. Bauerfeld, W., Diener, U., Ratge, D., Knoll, E. & Wisser, H. (1982) Fresenius Z. Anal. Chem. *311*, 436–437.
36. Speek, A. J., Odink, J., Schrijver, J. & Schreurs, W. H. P. (1983) Clin. Chim. Acta *128*, 103–113.
37. Hansson, L., Glad, M. & Hansson, C. (1983) J. Chromatogr. *265*, 37–44.
38. Kemper, K., Hagemeier, E., Boos, K.-S. & Schlimme, E. (1984) Fresenius Z. Anal. Chem. *317*, Heft 6.
39. Weith, H. L., Wiebers, J. L. & Gilham, P. T. (1970) Biochemistry *9*, 4396–4401.
40. Moore, E. C., Peterson, D., Ying Yang, L., Yau Yeung, C. & Faye Neff, N. (1974) Biochemistry *13*, 2904–2907.
41. Singhal, R. P., Bajaj, R. K., Buess, C. M., Smoll, D. B. & Vakharia, V. N. (1980) Anal. Biochem. *109*, 1–11.
42. Ackermann, S., Cool, B. & Furth, J. (1979) Anal. Biochem. *100*, 174–178.

Prof. Dr. Dr. Eckhard Schlimme
Laboratorium für Biologische Chemie
Universität-GH-Paderborn
Warburger Straße 100
D-4790 Paderborn